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Mapping the protein regions responsible for the functional specificities of the *Arabidopsis* MADS domain organ-identity proteins

(flower development/domain swapping)

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ABSTRACT **The *Arabidopsis* MADS domain proteins AP1, AP3, PI, and AG specify floral organ identity. All of these proteins contain a MADS domain required for DNA binding and dimerization; a region termed L (linker between MADS domain and K domain), which plays an important role in dimerization specificity; the K domain, named for its similarity to the coiled-coil domain of keratin; and a C-terminal region of unknown function. To determine which regions of these proteins are responsible for their abilities to specify different organs, we have made a number of chimeric MADS box genes. The *in vivo* function of these chimeric genes was investigated by ectopic expression in transgenic *Arabidopsis* plants. The four proteins fall into two classes on the basis of regions responsible for their functional specificities. The L region and K domain define the functional specificities of AP3 and PI, while the MADS domain and L region define the functional specificities of AP1 and AG.**

One general question in the molecular analysis of development is how homeotic genes, which are key regulators of organ or body-segment identity, perform their functions. Many homeotic genes have been cloned and shown to encode DNA-binding proteins. While these proteins are thought to act by binding to the promoters of different genes and thus regulating the spatial and temporal expression patterns of these genes, the mechanism leading to the activation or repression of specific genes is often unknown, as is (for the most part) the identity and function of the downstream genes. In some cases, a group of closely related homeotic genes act to specify different developmental pathways in adjacent regions. Two examples are homeotic selector genes in insects and vertebrates, which encode DNA-binding homeodomain proteins that act in different segments to specify different fates (reviewed in refs. 1 and 2), and organ-identity genes in flowers, which encode DNA-binding MADS domain proteins that act to specify organ primordia as sepals, petals, stamens, or carpels (reviewed in refs. 3–5).

In the case of homeotic genes that are members of gene families yet have different functions *in vivo*, a way to investigate the mode of action of their gene products is to identify the particular region(s) of the proteins that is responsible for the different organ-specifying activities. This can be done by making chimeric genes, fusing parts from proteins that are related but have different developmental functions, and then assaying the *in vivo* function of the chimeric proteins. If the DNA-binding regions are implicated, then the different functions of the proteins may be a result of intrinsic differences in sequence specificity conferred by their related but nonidentical DNA-binding domains. This would cause the proteins to bind different DNA sequences and regulate different sets of genes. Downstream genes could then be identified by determination of the binding-site selectivities of the proteins by using *in vitro* techniques, followed by identification of these sequences in the genome. Alternatively, the DNA-binding domains could all be equivalent, with other protein regions responsible for the different functions of the related proteins. In this case, an understanding of the specificity of these regulators will likely lie in understanding their interactions with other proteins, which may either direct the regulators to different genomic regions or dictate different functions of the homeotic proteins when they are bound to the same genomic sequences.

Here, we have made chimeric proteins based on four different plant MADS domain proteins [APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), and AGAMOUS (AG)], each with a similar structure but different functions in floral organ specification. Several years ago a genetic model was developed in which the activities of these four proteins were assigned to three different classes, A, B, and C, with AP1 an A function protein, AP3 and PI required for B function, and AG required for C function (6–9). The proteins were proposed to function combinatorially in the specification of floral organs, such that A function specifies sepals, A function in combination with B function specifies petals, B and C functions together specify stamens, and C function specifies carpels. All of these proteins are members of the MADS domain family of transcription factors which includes proteins from yeast (MCM1), animals (SRF, MEF2), and plants (reviewed in ref. 10). They share a conserved 56-amino acid DNA-binding and dimerization motif called the MADS domain. The four plant proteins also share an additional region, the K domain, which has low similarity at the amino acid level but is predicted to form amphipathic α-helices in each protein (11, 12). The region between the MADS and K domains is called the L, or linker, domain.

Ectopic expression of each of the four MADS box genes under the control of the constitutive 35S cauliflower mosaic virus promoter (p35S) produces a distinctive dominant gain-of-function phenotype (13–17). The uniqueness of these ectopic expression phenotypes has allowed us to assay the ability of chimeric MADS box genes to act like their respective parental genes in transgenic *Arabidopsis* plants. We find that the functional specificities of these four proteins fall into two classes with the functional specificity of AP3 and PI dependent on their L and K regions, while the specificity of AG and AP1

Abbreviations: AP1, APETALA1; AP3, APETALA3; PI, PISTILLATA; AG, AGAMOUS.

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resides in their MADS and L domains. These differences may result from different protein–protein interaction surfaces and/or different MADS-domain dimerization requirements but do not implicate different intrinsic DNA-binding properties in functional specificity.

MATERIALS AND METHODS

Construction of Chimeric Genes. The chimeric MADS box genes were constructed by using PCR mutagenesis, involving two rounds of PCR. In the first round, one outer primer (containing either a *Bam*HI or an *Xba* I site) and one internal primer (containing sequences from both of the MADS box parental genes to be fused) were used. The products of these two PCR reactions were then used in a second PCR reaction with the outer primers. All constructs were ligated into the *Bam*HI and *Xba* I sites of pGEM-3Z into which an 842-bp 35S promoter had previously been inserted in the *Asp*718 and *Bam*HI sites. Correct sequences were confirmed by double-stranded sequencing of the recombinant plasmids. The 35S promoter–chimeric MADS genes were then cloned into the *Asp*718 and *Xba* I sites of pCGN1547 (18) containing a 253-bp sequence from the 3′ end of nopaline synthase in the *Xba* I and *Pst* I sites.

Agrobacterium-Mediated Transformation. Agrobacterium strain ASE was transformed with the chimeric genes in the pCGN1547 vector. Transformed Agrobacterium was used to transform *Arabidopsis* plants by standard root methods into No-0 (BGFN8 and -11) (19) or by vacuum infiltration into L-*er* [BGFN2, -3, -5, -13, -19, -20, -22, -23, -29, and p35S–AG(ML), p35S–AP1(ML)] (20). Transformants were selected by plating the seeds on kanamycin plates. DNA gel blots, performed on six lines of BGFN13, indicated that all six lines arose from independent insertions.

Scanning Electron Microscopy. Samples were fixed, dried, coated, and dissected as described (6, 21). The images were photographed with Kodak TMAX film.

Strain Constructions. BGFN plants were crossed to homozygous *ap3–3*, *pi-1*, *ap1–1*, heterozygous *ag-3*, and transgenic p35S–AP3 (15) and p35S–PI (17) plants by manual cross-pollination. The genotype of BGFN22 *ap1–1* plants was confirmed by sequencing DNA of PCR reactions of leaf tissue (22) which amplified *AP1*.

RESULTS

The amino acid sequences of AP3, PI, AG, and AP1 are shown in Fig. 1*A*. All of these proteins contain a MADS domain; an

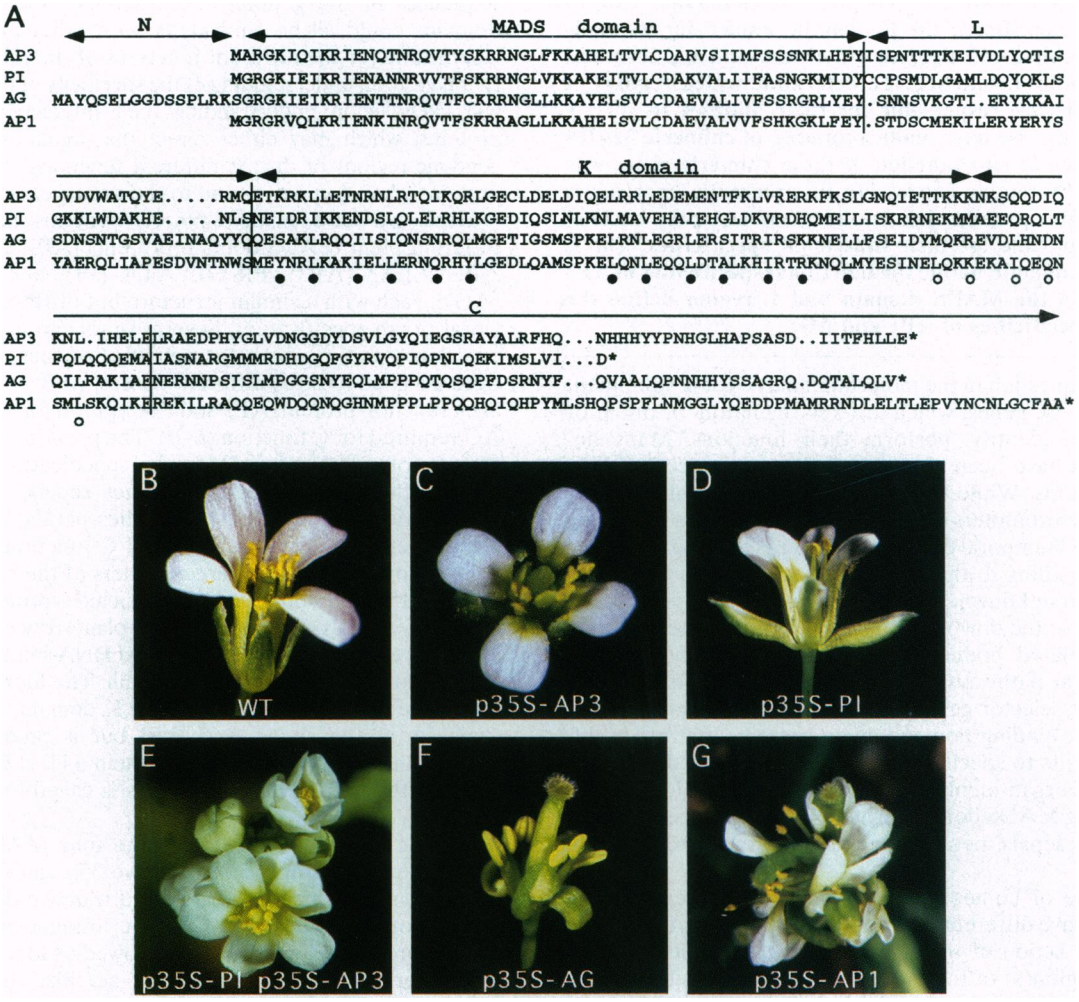

FIG. 1. Sequences of AP3, PI, AG, and AP1 and phenotypes of flowers resulting from ectopic expression of these genes. (*A*) Sequence alignment of AP3, PI, AG, and AP1. The N (part of the N-terminal region of AG), MADS domain, L, K domain, and C (C-terminal) regions are indicated by arrows. The sequence shown for AG is the same as that used in p35S–AG (14) in which a Thr has been mutated to become the Met start codon. Solid circles mark the hydrophobic *a* and *d* positions of the proposed K domain coiled coil (11). The sites at which sequences from these four proteins were fused to make the chimeric MADS box genes are indicated by vertical lines. The site of fusion between the K domain and C-terminal region was chosen somewhat C terminal to the indicated K domain since a third helix (indicated by open circles) has been proposed in some plant MADS domain proteins (23). (*B*) Wild-type *Arabidopsis* flower. (*C*) p35S–AP3 flower. (*D*) p35S–PI flower. (*E*) p35S–PI p35S–AP3 flower. (*F*) p35S–AG flower. (*G*) p35S–AP1 flower.

L region that plays a role in determining which MADS domain dimers are capable of DNA binding (35); the K domain, a region proposed to form amphipathic α-helices (11, 12); and a nonconserved C-terminal sequence (C). The positions at which the various sequences were fused (indicated by vertical lines in Fig. 1*A*) are located at the end of the MADS domain, at the end of the L region, and between the K domain and C-terminal region. A summary of the chimeric constructs and their ectopic expression phenotypes is shown in Fig. 2. A more complete list of these phenotypes is found in Table 1. The chimeric constructs in Fig. 2 and Table 1 are arranged in the order in which they will be discussed.

The phenotypes resulting from ectopic expression of *AP3*, *PI*, *AG*, and *API* (Fig. 1 *B–G*) will be described here briefly for comparison with the ectopic expression phenotypes of the chimeric MADS box genes. p35S–AP3 flowers exhibit a partial transformation of fourth-whorl carpels into stamens (15) (Fig. 1*C*), while p35S–PI flowers exhibit a partial transformation of first-whorl sepals into petals (17) (Fig. 1*D*). Ectopic expression of both B-class genes together results in flowers with petals in whorls one and two and stamens in whorls three and four (17) (Fig. 1*E*). Ectopic expression of *AG* (p35S–AG) results in flowers with a range of phenotypes. Severely affected flowers have carpelloid first-whorl organs and missing or staminoid second-whorl organs, while less affected flowers have sepals with some stigmatic papillae and small petals (14) (Fig. 1*F*). p35S–AG plants exhibit other phenotypes, including curled leaves and early termination of the inflorescence in carpels or carpelloid structures (14). p35S–AP1 plants produce only a few normal-looking flowers before the apical and lateral inflorescences terminate in a flower or a floral structure with an abnormal number and pattern of organs (16) that resemble *tfl* mutants (24, 25) (Fig. 1*G*).

The Functional Specificity of AP3 Is Determined by the L Region and the K Domain. The chimeric construct *BGFN1* contains the *AG* MADS box and the *AP3* L, K, and C regions (Fig. 2). Ectopic expression of *BGFN1* results in flowers resembling those of p35S–AP3 plants, in which the fourth-whorl carpels are replaced with stamens and carpelloid stamens. A fuller description of these lines will be published elsewhere (B.A.K., J. L. Riechmann, and E.M.M., unpublished results). To refine the protein domains responsible for the specificity of AP3, another chimeric gene, *BGFN11*, was made. *BGFN11* has the *AG* MADS box fused to the *AP3* L region and K box and the *AG* C-terminal region (Fig. 2). Nine of 13 BGFN11 lines exhibited a p35S–AP3 phenotype with stamens and carpelloid stamens present in the fourth whorl (compare Fig. 3 *A* and *B* with Fig. 1*C*), while the remaining four lines have wild-type flowers. The ability of *BGFN11* to substitute for *AP3* was further investigated by observing *BGFN11* in an *ap3–3* mutant background and in a p35S–PI background. *ap3–3* flowers have two outer whorls of sepals and two inner whorls of carpels (26). BGFN11 *ap3–3* flowers have a phenotype similar to p35S–AP3 *ap3–3* flowers with whorls composed of sepals (first), sepaloid petals (second), stamens (third), and stamens or carpelloid stamens (fourth) (15) (Fig. 3*C*). BGFN11 p35S–PI plants have flowers with petals in whorls 1 and 2 and stamens in whorls 3 and 4 and are indistinguishable from p35S–PI p35S–AP3 flowers (compare Fig. 3*D* with Fig. 1*E*). Thus, the AP3 L region and K domain are sufficient to specify the AP3 function in an AG context.

Replacement of the L region (*BGFN2*) or K box (*BGFN5*) of *AP3* with the corresponding sequences from *AG* results in chimeric genes that when ectopically expressed do not behave like *AP3*. Almost all (27/29) of the BGFN2 lines exhibit a wild-type phenotype, except for one line which has some flowers containing extra carpels and one line with sepaloid

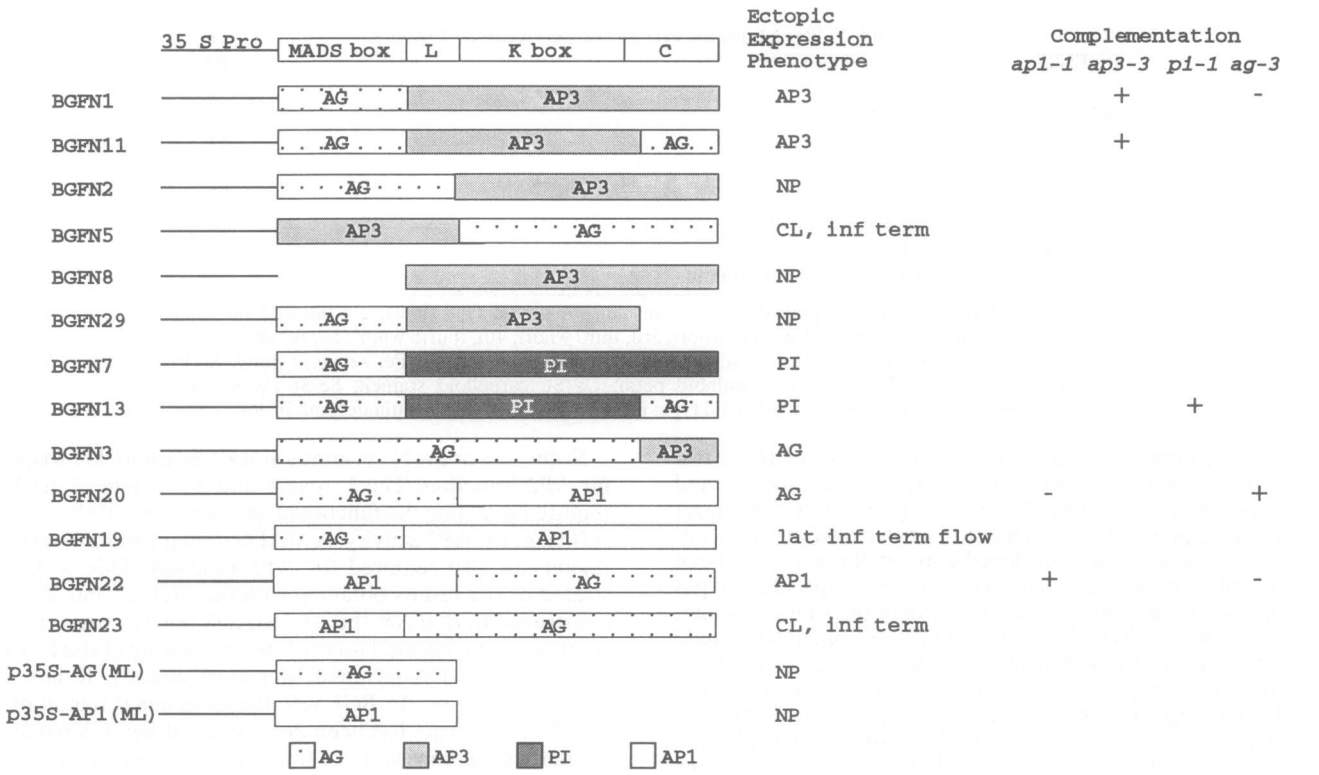

FIG. 2. Summary of the chimeric MADS box genes, the phenotypic effects of ectopic expression, and the results of complementation analyses. CL, curled leaves; inf. term., inflorescence terminates early; lat. inf. term. flow., lateral inflorescences are converted to terminal flowers; and NP, no phenotype. (*Left*) Schematic representation of the chimeric constructs. (*Center*) Phenotypes of *Arabidopsis* plants ectopically expressing the chimeric genes. (*Right*) Complementation analyses. The ability (+) or inability (−) to complement the *ap1–1*, *ap3–3*, *pi-1*, or *ag-3* mutations to a degree similar to that of the full-length parental gene under the control of the 35S promoter is indicated.

Table 1. Phenotypes of T_1 transformants

Construct	Phenotypes	No. of lines
BGFN11	4th: St, Ca/St	9
	WT	4
BGFN2	WT	27
	2nd: Se/Pe; 3rd: some Ca/St	1
	4th: some extra Ca	1
BGFN5	some CL; WT flowers	18
	CL; 1st: thin Se, Ca/Se; 2nd: thin Pe; 4th: crinkled Ca; inf term	5
	CL; 1st: thin and/or pointed Se; 2nd: thin Pe	4
	CL; inf term	3
	some CL; 4th: crinkled Ca	4
	CL; indeterminate	1
BGFN8	WT	10
	2nd: Se or Se/Pe	6
	2nd: narrow Pe	1
BGFN29	WT	16
	2nd: Se/Pe, some with 3rd: carpelloid organs	7
BGFN13	1st: Pe/Se	43
	1st: slightly Pe/Se	13
	WT	10
	1st: Pe/Se; 2nd: Se/Pe	5
	2nd: Se/Pe	5
	1st: slightly Pe/Se; 4th: partly indeterminate St & Ca inside Ca	1
BGFN3	CL; 1st: some Ca/Se; 2nd: St/Pe; inf term	40
	1st: some pointed Se; 2nd: Se-St/Pe; 4th: some extra Ca	8
	some CL; WT flowers	8
	1st: some pointed Se; 2nd: Se/Pe or St/Pe	9
	some CL; 2nd: narrow Pe; 4th: some extra Ca	5
	2nd: Se; indeterminate; 4th: Ca (with organs inside) or Se-St/Ca	1
BGFN20	CL; 1st: Ca/Se in later flowers; 2nd: St/Pe; inf term	7
	some CL; WT flowers	6
	CL; 2nd: narrow Pe	3
BGFN19	CL; lateral inf become flower/terminal flower	6
	CL; WT flowers	6
	WT	3
	CL; 2nd: St/Pe; lateral inf become flower/terminal flower	1
BGFN22	CL; terminal flower	10
	WT	7
BGFN23	CL; inf term	15
	CL; WT flowers	12
	WT	7
p35S-AG(ML)	WT	27
	2nd: slightly small Pe	1
p35S-AP1(ML)	WT	18
	slightly CL; WT flowers	1

Only the whorls containing organs different from those in a wild-type flower are listed in the table. Abbreviations: 1st, first whorl; 2nd, second whorl; 3rd, third whorl; 4th, fourth whorl; Se, sepal; Pe, petal; St, stamen; Ca, carpel; Pe/Se, petaloid sepal; Ca/Se, carpelloid sepal; Se/Pe, sepaloid petal; St/Pe, staminoid petal; Se-St/Pe, sepaloid staminoid petal; Ca/St, carpelloid stamen; Se-St/Ca, sepaloid staminoid carpel; CL, curled leaves; WT, wild type; inf term, inflorescence terminates; inf, inflorescence.

petals and carpelloid stamens. The phenotypes of these two lines somewhat resemble plants heterozygous for *ag* and mutant for *ap3*, respectively, indicating that they may result from cosuppression. Cosuppression or inactivation of an endogenous gene by an introduced copy of the gene has been observed in plants (27, 28). Cosuppression-type phenotypes were occasionally observed with several of the chimeric genes. The inability of *BGFN2* to confer an ectopic phenotype could result from the formation of a completely inactive chimeric protein or a chimeric protein that is partially functional but which no longer retains the ability to specify *AP3* function. *In vitro* data have shown that BGFN2 is capable of binding to DNA as a homodimer, indicating that the protein is at least partially functional (35) and provides evidence that the AP3 L region is required for AP3 specificity. Ectopic expression of *BGFN5* results in phenotypes (to be described later) that are not characteristic of p35S–AP3. This suggests that the AP3 K domain is also required for AP3 functional specificity.

Sequences at the N Terminus and C Terminus Are Required for AP3 Function. The L region and K domain of AP3 are required to define the functional specificity of AP3 but are not sufficient for AP3 activity. A MADS domain and a C-terminal region are also required for AP3 function. This is demonstrated by the results obtained with the *BGFN8* and *BGFN29* constructs. In *BGFN8*, the *AP3* MADS box has been deleted, resulting in a truncated form of *AP3* consisting of the L region (with an added N-terminal Met start codon), K box, and C-terminal region. In *BGFN29*, the C-terminal region of the *BGFN11* construct has been deleted, resulting in a truncated chimeric gene consisting of the *AG* MADS box fused to the *AP3* L and K box regions. Neither is able to function as *AP3 in vivo*, although BGFN29 was capable of interacting with PI to bind DNA (unpublished observations). Ten of the 17 BGFN8 lines have a wild-type appearance, while 6 of the lines have sepals or sepaloid petals in the second whorl of the

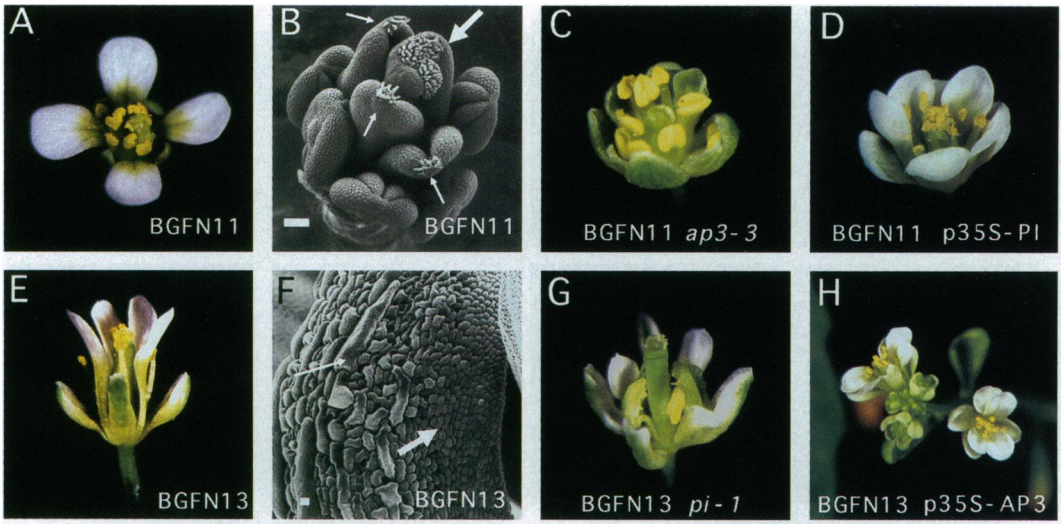

FIG. 3. Functional specificity of AP3 and PI. (*A*) BGFN11 flower containing stamens and a carpelloid organ in the center of the flower. (*B*) Scanning electron micrograph of BGFN11 flower containing stamens topped with stigmatic tissue (indicated by thin white arrows) and a carpelloid organ in the center of the flower (indicated by a thick white arrow). (Bar = 100 μm.) (*C*) Flower of a BGFN11 *ap3-3* plant with sepals in the first whorl, sepaloid petals in the second whorl, stamens in the third whorl, and stamens and carpelloid stamens in the fourth whorl. (*D*) BGFN11 p35S–PI flower with petals in whorls 1 and 2 and stamens in whorls 3 and 4. (*E*) BGFN13 flower with petaloid sepals in the first whorl. (*F*) Scanning electron micrograph of BGFN13 flower showing the mosaic nature of these first-whorl organs. Small round petal cells are located on the edge of the organ (indicated by thick white arrow) and longer, more irregularly shaped sepal cells are found in the interior region of the organ (indicated by thin white arrow). (Bar = 10 μm.) (*G*) BGFN13 *pi-1* flower with petaloid sepals in the first whorl, petals in the second whorl, stamens and carpelloid stamens in the third whorl, and carpels in the fourth whorl. (*H*) BGFN13 p35S–AP3 flower with petals in whorls 1 and 2 and stamens in whorls 3 and 4.

flower. For BGFN29, 16 of the 23 lines had wild-type flowers, while 7 had sepaloid petals with some of these also having carpelloid organs in the third whorl. The inability of BGFN29 to act like AP3 indicates that the C-terminal sequence of AP3 has an important function. Although this region exhibits no sequence homology between AG and AP3, the ability of the AG C-terminal sequence to substitute for that of AP3 (BGFN11) suggests that this region has a similar function in both proteins.

The Functional Specificity of PI Is Determined by the L Region and the K Domain. Ectopic expression of *BGFN7*, which contains the *AG* MADS box fused to the L, K box, and C regions of *AP3* (Fig. 2), produces flowers with sepaloid petals in the first whorl, which are identical to those of p35S–PI flowers (17). A fuller description of BGFN7 lines will be published elsewhere (B.A.K., J. L. Riechmann, and E.M.M., unpublished results). Ectopic expression of *BGFN13*, which consists of the *AG* MADS box and C-terminal region fused to the L and K regions of *PI* (Fig. 2), results in the same first-whorl petaloid sepal phenotype as exhibited by p35S–PI plants (compare Fig. 3 *E* and *F* with Fig. 1*D*). More than 60 of 77 lines exhibited this first-whorl phenotype (Table 1). Thus, the functional specificity of PI is conferred by the L and K regions. The ability of *BGFN13* to function as *PI* was investigated further by crossing plants containing this construct into *pi-1* and p35S–AP3 plants. *pi-1* flowers have sepals in whorls 1 and 2 and an abnormally large gynoecium in the fourth whorl (21). BGFN13 *pi-1* plants have petaloid sepals in the first whorl; petals in the second whorl; stamens, carpelloid stamens, carpelloid organs, and filaments in the third whorl; and carpels in the fourth whorl (Fig. 3*G*). The number of third-whorl organs is often less than six. Sometimes, the fourth-whorl carpels are fused to third-whorl organs or are misshapen. *BGFN13* is thus able to rescue the second-whorl organs of *pi-1* but is not able to fully rescue the third-whorl organs. This is similar to what is seen in p35S–PI *pi-1* flowers (unpublished results). Plants ectopically expressing both *BGFN13* and *AP3* have petals in whorls 1 and 2 and stamens in whorls 3 and 4 and, thus, look like flowers of p35S–PI p35S–AP3 plants (compare Fig. 3*H* with Fig. 1*E*).

The Functional Specificity of AG Is Determined by the MADS Domain and the L Region. Ectopic expression of the chimeric gene *BGFN3*, in which the MADS box, L region, and K box of *AG* were fused to the C-terminal region of *AP3* (Fig. 2), resulted in a p35S–AG phenotype (compare Fig. 4*B* with Fig. 1*F*). More than 40 of the 71 lines generated exhibited characteristics of ectopic *AG* expression, including dwarfed plants, curled leaves, carpelloid sepals (Fig. 4*A*), staminoid petals (Fig. 4*B*), and early termination of the inflorescence meristem (Fig. 4*A*). The transformed lines exhibited a range in the severity of these phenotypes, with some lines producing only two or three flowers before the inflorescence terminated. The remaining 31 lines had a variety of phenotypes (Table 1), including wild-type flowers or flowers with narrow petals or sepaloid petals in the second whorl.

Another construct created to test *AG* function (*BGFN20*) contains the *AG* MADS box and L region fused to the K box and C-terminal region of *AP1* (Fig. 2). For BGFN20, 14 of the 16 lines exhibited curled leaves (Fig. 4*C*). The effect of ectopic expression of *BGFN20* on flowers was variable. Seven of 16 lines had staminoid petals (Fig. 4 *D* and *E*), while 3 additional lines had narrow petals. The sepals of later-produced flowers were often carpelloid (Fig. 4*F*), and the inflorescences of these plants often terminated early in carpelloid structures (Fig. 4*G*). All of these are characteristics of *AG* and not *AP1* function. *BGFN20* exhibited partial rescue of an *ag-3* mutant flower. BGFN20 *ag-3* flowers are indeterminate and consist of organs in the repeating pattern: (sepal or carpelloid sepal, staminoid petal, staminoid petal)$_n$ (Fig. 4*H*) instead of the usual (sepal, petal, petal)$_n$ pattern of *ag-3* flowers (6). Stigmatic tissue is sometimes present on the sepals of BGFN20 *ag-3* flowers. p35S–AG *ag-1* plants produce indeterminate flowers consisting of carpelloid sepals and narrow and yellowish petals (Yukiko Mizukami and Hong Ma, personal communication). Thus, *BGFN20* is able to rescue the *ag* mutant phenotype to a degree similar to that of full-length *AG* expressed from a 35S promoter, indicating that the MADS domain and L region of AG are sufficient to specify AG function in an AP1 context.

Ectopic expression of *BGFN2*, a construct which consists of the *AG* MADS box and L region fused to the K box and

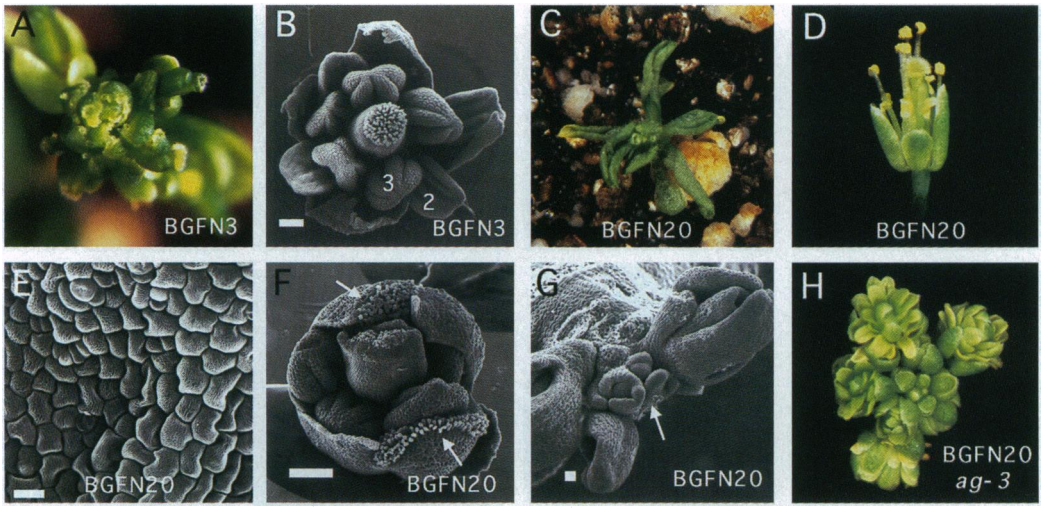

FIG. 4. Functional specificity of AG. Numbers indicate the whorl. (*A*) BGFN3 flower exhibiting carpelloid first-whorl organs and early termination of the inflorescence. (*B*) Scanning electron micrograph of BGFN3 flower with staminoid petals in the second whorl. (Bar = 100 μm.) (*C*) BGFN20 plant exhibiting curled leaves. (*D*) BGFN20 flower with staminoid petals in the second whorl. (*E*) Scanning electron micrograph of the abaxial surface of a staminoid petal of a BGFN20 flower. The shape of the cells is characteristic of cells found on the surface of anthers. (Bar = 10 μm.) (*F*) Scanning electron micrograph of a later-produced BGFN20 flower with carpelloid first-whorl organs (indicated by white arrows). (Bar = 100 μm.) (*G*) Scanning electron micrograph of the inflorescence of a BGFN20 plant. The meristem (indicated by white arrow) stops producing flowers and usually terminates in carpelloid structures. (Bar = 10 μm.) (*H*) BGFN20 *ag-3* flowers, which are indeterminate and consist of sepals, carpelloid sepals, and staminoid petals.

C-terminal region of *AP3* (Fig. 2) does not result in a p35S–AG phenotype. *BGFN2* contains the same *AG* MADS box and L region sequence that is present in *BGFN20*. The only difference between the two chimeric genes is that in *BGFN20* the *AG* sequences are fused to the *AP1* K box and C region, while in *BGFN2* the *AG* sequence is fused to *AP3* K box and C region.

The MADS domain of AG is unable to specify AG functional specificity in either an AP3 (BGFN1) or AP1 (BGFN19) context. The *BGFN19* construct contains the *AG* MADS box fused to the L, K, and C regions of *AP1*. Six of 16 lines produced plants in which lateral but not apical inflorescences were converted to flowers, indicating that these lines possess partial *AP1* function (16). A single line had characteristics of both ectopic *AP1* and ectopic *AG* function with lateral inflorescences converted to flowers (ectopic *AP1* function) and staminoid petals present in the second whorl (ectopic *AG* function). An additional six lines exhibited leaf curling but were otherwise wild type in appearance, while three lines were wild type in all respects.

The previously mentioned *BGFN5* chimeric gene consists of the *AP3* MADS box and L region fused to the *AG* K box and C region. Approximately half (18/35) of the lines exhibited no floral phenotype, although 10 of these lines had curled leaves similar to those found in plants ectopically expressing *AG*, *AP3* and *PI*; or *AP1*. Five of the lines exhibited a graded floral phenotype. The early-produced flowers were largely wild type, although the sepals and petals appeared thinner than in wild-type flowers. However, the inflorescence of these plants often terminated in a floral structure consisting mostly of stamens and carpels, and flowers produced by the inflorescence shortly before termination often had staminoid or carpelloid sepals.

The ability of *BGFN5* to act like *AG* was investigated by making BGFN5 *ag-3* plants. BGFN5 *ag-3* flowers are indeterminate, consisting of sepals and petals. However, in some of the BGFN5 *ag-3* plants, carpelloid tissue was occasionally observed on the sepals. Carpelloid tissue is never present on the sepals of *ag-3* flowers. These results suggest that the *BGFN5* construct may possess some functions characteristic of ectopic *AG*, including curled leaves, the premature termination of the inflorescence, and aspects of carpel identity. However, carpel features such as stigmatic tissue are present on the leaf-like organs of *ap2–2 ag-1 pi-1* flowers (6), and, thus, the presence of these features does not necessarily indicate *AG* function.

The Functional Specificity of AP1 Is Determined by the MADS Domain and the L Region. The construct *BGFN22*, in which the *AP1* MADS box and L region is fused to the K box and C-terminal region of *AG* (Fig. 2), confers a p35S–AP1 phenotype when ectopically expressed in *Arabidopsis* plants (compare Fig. 5*A* with Fig. 1*G*). Flowers on 10 of 17 lines exhibit a *tfl* mutant phenotype, in which the inflorescence produces only a few normal flowers before terminating in a

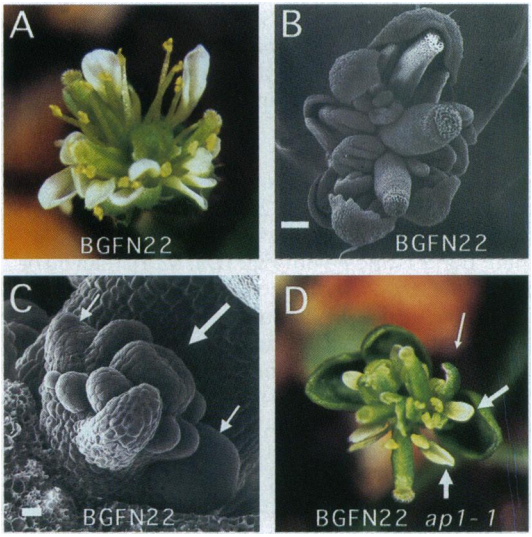

FIG. 5. Functional specificity of AP1. (*A*) BGFN22 terminal flower. (*B*) Scanning electron micrograph of a BGFN22 terminal flower. (Bar = 100 μm.) (*C*) Scanning electron micrograph of a young BGFN22 terminal flower. The inflorescence meristem has been converted to a floral meristem. Bulges (indicated by small white arrows) on the sides of this flower primordia (indicated by larger white arrow) correspond to additional floral meristems which will form part of the terminal flower structure. (Bar = 10 μm.) (*D*) BGFN22 *ap1–1* flower. Sepals (indicated by thin white arrow) and petals (indicated by thick white arrows) are present in these flowers.

floral structure in which several flowers appear to arise together (Fig. 5 *A–C*). These lines also had curled leaves. An additional seven lines had a wild-type appearance. p35S–AP1 rescues the strong *ap1–15* allele in the Columbia ecotype (16). BGFN22 is able to largely rescue *ap1–1* mutants. *ap1–1* flowers have leaf-like first-whorl organs, are almost always missing second-whorl organs, have a reduced number of third-whorl stamens, have a normal fourth-whorl of carpels, and develop flowers in the axils of the first-whorl organs (29, 30). BGFN22 *ap1–1* flowers have sepals and petals, although the number is reduced compared with wild type (Fig. 5*D*). Axillary flowers were only occasionally seen. Thus, the MADS and L regions of *AP1* (in an *AG* context) are sufficient to specify the *AP1* function.

A construct, *BGFN23*, in which the MADS box of *AP1* was fused to the L, K box, and C regions of *AG*, demonstrates that the *AP1* MADS box alone in an *AG* context is not sufficient to specify *AP1* function. Fifteen lines exhibited curled leaves and had inflorescences which terminated early in carpelloid structures and resembled the BGFN5 lines described above. Another 12 lines had wild-type flowers and curled leaves, while the remaining 7 lines were wild type in appearance.

The MADS Domain and the L Region Are Not Sufficient to Confer AG and AP1 Function. The ability of the MADS and L regions alone to specify AG and AP1 function was investigated by ectopic expression of these regions behind the 35 S promoter. Twenty-seven lines of p35S–AG(ML) exhibited a wild-type appearance, while 1 line had flowers with petals that appeared slightly small. Eighteen lines of p35S–AP1(ML) were wild type, and one line had somewhat curled leaves. These results indicate that the MADS and L region are not sufficient to specify the AG and AP1 functions. A K domain and C region are also required for AG and AP1 function.

DISCUSSION

Studies of a series of chimeric MADS box genes have enabled us to map the regions responsible for the functional specificities of the MADS domain proteins AP1, AP3, PI, and AG. The specificities of these four proteins reside in different but overlapping regions. The L region and K domain contain the regulatory specificities of AP3 and PI, while the MADS domain and L region determine the functional specificity of AP1 and AG (Fig. 6). The functional specificity domains that we have defined may be context dependent (as demonstrated by BGFN20 and BGFN2) and certainly may be smaller than indicated here because only exchanges of large domains have been tested. In the case of AG, the N-terminal half of the MADS domain does not seem to be required for AG specificity, as demonstrated by additional chimeric MADS box genes (unpublished results).

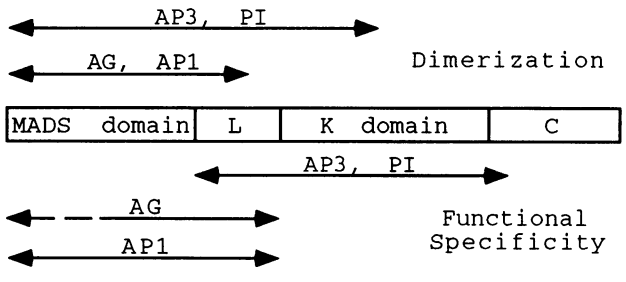

FIG. 6. Differences in the functional-specificity domains and dimerization requirements of AP3, PI, AP1, and AG. The functional specificities of AP3 and PI have been mapped to the L and K domain regions, while the functional specificities of AP1 and AG have been mapped to the MADS domain and L region. On the basis of results from additional chimeric MADS genes in which the N-terminal half of the AG MADS domain was replaced with the corresponding sequence of PI or AP3 (unpublished results), the functional specificity domain of AG can probably be narrowed down to the C-terminal half of the MADS domain and the L region. The smallest "core" sequence which will dimerize and bind DNA for each of these four proteins is indicated at the top of the figure.

Different Functional-Specificity Domains. *In vivo* experiments with chimeric MADS box genes in which the MADS domain or N-terminal half of the MADS domain were switched between proteins have indicated that the intrinsic DNA-binding specificities of AG, AP3/PI, and AP1 complexes are quite similar (unpublished results). This evidence suggests that the regulatory specificities of these proteins do not result from different DNA-binding specificities but perhaps from interactions with different accessory proteins. This is emphasized by the results given here, which show that the functional specificity of AP3 and PI does not depend on the DNA-binding MADS domain and that that of AG and AP1 depends on the L region as well as the MADS domain. The differences observed in the functional specificity domains could result from different accessory protein interaction surfaces. From the results described here, one would expect the interaction regions of AP3 and PI to be the L and K domains and those of AG and AP1 to be the MADS and L domains. Characterization of the protein–protein interactions involving other MADS domain family members has indicated that the MADS domain and the region directly following it are used to interact with cofactors. The interaction between the MADS domain protein MEF2 (muscle enhancer factor 2) and the basic helix-loop-helix proteins MyoD/E12 involves both the MADS domain and the MEF2 domain which follows the MADS domain (31). MCM1 interacts with at least three proteins (α1, α2, and STE12) by using residues at the C-terminal end of the MADS domain and extending past it (32–34).

An additional explanation for the observed differences in the domains required for functional specificity is the different MADS domain dimerization requirements of AP1, AG, AP3, and PI. The smallest fragments of AP1 and AG which will dimerize and bind DNA are the MADS domain and part of the L region, while the N terminus of the K domain is required in addition to the MADS and L regions to form a DNA-binding AP3–PI heterodimer (J. L. Riechmann, and E.M.M., unpublished observations; Fig. 6). In all four cases, the functional-specificity domain includes the L region. It is an open possibility that this region is critical for interactions with accessory proteins, while the other regions of the functional-specificity domains (the MADS domain for AG and AP1 and the K domain for AP3 and PI) are required to achieve proper dimerization.

Context Dependency. The functional-specificity regions we have determined may be dependent upon the context in which they occur. The AG MADS box and L regions were sufficient in an AP1 context (BGFN20) to specify AG function but were not sufficient in an AP3 context (BGFN2). One explanation is that residues in the K domain of AG contribute to its functional specificity. Some evidence for this is provided by BGFN5, which contains the K and C regions of AG and may possess some ectopic AG functions. The ability of BGNF20 to act like AG suggests that the corresponding sequence in AP1 is capable of providing an equivalent function, while that in AP3 does not. The K domains of AG and AP1 are slightly more related than those of AG and AP3 on the basis of amino acid identity. A second possibility is that the K domain and C-terminal regions of AP3 inhibit the ability of the MADS domain and L region of AG to carry out their function.

We have found that the regions responsible for the functional specificities of the four MADS domain proteins, AP3, PI, AG, and AP1, map to different but overlapping regions. These differences may correspond to different dimerization requirements and/or different accessory-protein-interaction domains but do not seem to correspond to different intrinsic

DNA-binding specificities. The identification of accessory proteins and analysis of their interactions with these MADS domain proteins thus seems to be the way to understand the floral homeotic genes; studies of their intrinsic DNA-binding abilities would not be expected to answer questions concerning their specific functions.

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1. McGinnis, W. & Krumlauf, R. (1992) *Cell* **68,** 283–302.
2. Lawrence, P. A. & Morata, G. (1994) *Cell* **78,** 181–189.
3. Ma, H. (1994) *Genes Dev.* **8,** 745–756.
4. Weigel, D. & Meyerowitz, E. M. (1994) *Cell* **78,** 203–209.
5. Yanofsky, M. F. (1995) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 167–188.
6. Bowman, J. L., Smyth, D. R. & Meyerowitz, E. M. (1991) *Development (Cambridge, U.K.)* **112,** 1–20.
7. Coen, E. S. & Meyerowitz, E. M. (1991) *Nature (London)* **353,** 31–37.
8. Meyerowitz, E. M., Bowman, J. L., Brockman, L. L., Drews, G. N., Jack, T. J., Sieburth, L. & Weigel, D. (1991) *Development (Cambridge, U.K.), Suppl.* **1,** 157–167.
9. Mandel, M. A., Gustafson-Brown, C., Savidge, B. & Yanofsky, M. F. (1992) *Nature (London)* **360,** 273–277.
10. Shore, P. & Sharrocks, A. D. (1995) *Eur. J. Biochem.* **229,** 1–13.
11. Ma, H., Yanofsky, M. F. & Meyerowitz, E. M. (1991) *Genes Dev.* **5,** 484–495.
12. Pnueli, L., Abu-Abeid, M., Zamir, D., Nacken, W., Schwarz-Sommer, Zs. & Lifschitz, E. (1991) *Plant J.* **1,** 255–266.
13. Mandel, M. A., Bowman, J. L., Kempin, S. A., Ma, H., Meyerowitz, E. M. & Yanofsky, M. F. (1992) *Cell* **71,** 133–143.
14. Mizukami, Y. & Ma, H. (1992) *Cell* **71,** 119–131.
15. Jack, T., Fox, G. L. & Meyerowitz, E. M. (1994) *Cell* **76,** 703–716.
16. Mandel, M. A. & Yanofsky, M. F. (1995) *Nature (London)* **377,** 522–524.
17. Krizek, B. A. & Meyerowitz, E. M. (1996) *Development (Cambridge, U.K.)* **122,** 11–22.
18. McBride, K. E. & Summerfelt, K. R. (1989) *Plant Mol. Biol.* **14,** 269–276.
19. Valvekens, D., Van Montagu, M. & Van Lijsebettens, M. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5536–5540.
20. Bechtold, N., Ellis, J. & Pelletier, G. (1993) *C.R. Acad. Sci.* **316,** 1194–1199.
21. Bowman, J. L., Smyth, D. R. & Meyerowitz, E. M. (1989) *Plant Cell* **1,** 37–52.
22. Klimyuk, V. I., Carroll, B. J., Thomas, C. M. & Jones, J. M. (1993) *Plant J.* **3,** 493–494.
23. Tröbner, W., Ramirez, L., Motte, P., Hue, I., Huijser, P. Lönnig, W, Saedler H., Sommer, H. & Schwarz-Sommer, Z. (1992) *EMBO J.* **11,** 4693–4704.
24. Shannon, S. & Meeks-Wagner, D. R. (1991) *Plant Cell* **3,** 877–892.
25. Alvarez, J., Guli, C. L., Yu, X. H. & Smyth, D. R. (1992) *Plant J.* **2,** 103–116.
26. Jack, T., Brockman, L. L. & Meyerowitz, E. M. (1992) *Cell* **68,** 683–687.
27. Napoli, C., Lemieux, C. & Jorgensen, R. (1990) *Plant Cell* **2,** 279–289.
28. van der Krol, A. R., Mur, L. A., Mol., J. N. M. & Stuitje, A. R. (1990) *Plant Cell* **2,** 291–299.
29. Irish, V. F. & Sussex, I. M. (1990) *Plant Cell* **2,** 741–753.
30. Bowman, J. L., Alvarez, J., Weigel, D., Meyerowitz, E. M. & Smyth, D. R. (1993) *Development (Cambridge, U.K.)* **119,** 721–743.
31. Molentin, J. D., Black, B. L., Martin, J. F. & Olsen, E. N. (1995) *Cell* **83,** 1125–1136.
32. Mueller, C. G. F. & Nordheim, A. (1991) *EMBO J.* **10,** 4219–4229.
33. Primig, M., Winkler, H. & Ammerer, D. (1991) *EMBO J.* **10,** 4209–4218.
34. Bruhn, L. & Sprague, G. F. (1994) *Mol. Cell. Biol.* **14,** 2534–2544.
35. Riechmann, J. L., Krizek, B. A. & Meyerowitz, E. M. (1996) *Proc. Natl. Acad. Sci. USA*, in press.